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Aichi Virus IgG Seroprevalence in Tunisia Parallels Genomic Detection and Clinical Presentation in Children with Gastroenteritis[▽]

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Aichi virus has been described as a novel causative agent of gastroenteritis in humans. In this study, we report the seroprevalence distribution of Aichi virus in Tunisia. A panel of 1,000 sera was screened by applying an enzyme-linked immunosorbent assay for immunoglobulin G specific for Aichi virus. A considerable prevalence (92%) of antibody to Aichi virus was found across all age groups. The specific anti-Aichi virus antibodies increased with age, from a high rate (68.8%) in children under 10 years old to about 100% in persons more than 60 years old. We found a statistically significant increase in levels of antibody to Aichi virus according to the age of patients. Immunoglobulin M antibodies were detected among five children. A high frequency of Aichi virus monoinfections in hospitalized children with severe gastroenteritis was previously observed in Tunisia. Aichi virus causes diarrhea with dehydration, fever, and vomiting. This work is the first to establish a correlation between the high seroprevalence of specific Aichi virus antibodies, clinical presentation, and a high frequency of isolation of Aichi virus by genomic characterization in stools of children suffering from gastroenteritis. Our data show the importance and emerging character of Aichi virus in the viral etiology of pediatric gastroenteritis.

Viral gastroenteritis is a common illness that affects humans worldwide. Rotavirus, calicivirus, adenovirus, and astrovirus have been established as the most important etiologic agents in these clinical diseases (4). Nevertheless, for many nonbacterial gastroenteritis cases, no etiological agent is diagnosed, and it has been hypothesized that other, viral agents are involved. Among these, Aichi virus (AiV) was first recognized in 1989 as the likely cause of oyster-associated gastroenteritis in a Japanese patient (15). This virus is a new member of the family *Picornaviridae* and is classified in a new genus, *Kobuvirus* (9, 15). The detection of AiV in stool samples collected from nonbacterial-gastroenteritis outbreaks due to oyster consumption was documented in Asia (14, 16, 19) and in Europe (1, 7). Moreover, this virus was recently identified in oysters implicated in a gastroenteritis outbreak in France (6). The detection of AiV strains has also been reported in fecal specimens from children suffering from gastrointestinal symptoms in several studies in Asian countries (8, 17, 20), in Brazil (7), and in France (1). However, a low incidence (0.9 to 3.1%) of AiV strains was observed in all these surveys in sporadic, as well as epidemic, gastroenteritis. On the other hand, several seroprevalence studies of antibodies to AiV have been conducted in Japan (16), Germany (7), and France (5) showing a high level of seroprevalence (80 to 95%) in adults, which supports widespread exposure to AiV, at least during childhood, and proves that the virus is quite prevalent.

In Tunisia, we previously reported the epidemiology and genomic characterization of AiV strains circulating in the pediatric Tunisian population over more than 4 years (12). In this previous prospective survey, contrary to the data in the literature, we showed that AiV was the third most frequently detected agent, after rotavirus and norovirus, in children with sporadic gastroenteritis symptoms. In addition, we observed a high incidence of monoinfections and a relatively high frequency of hospitalizations due to AiV infections, indicating the role of AiV as a causative agent of pediatric diarrhea in our country. Moreover, we previously analyzed sewage and shellfish samples for the presence of AiV from January 2003 to April 2007 (unpublished data), and we performed a comparative analysis of environmental AiV strains with those from clinical cases detected in the same period. AiV was the second most frequent pathogen in sewage, after rotavirus, and a correlation between environmental and human strains was observed. These previous data suggest that AiV plays an important role in pediatric gastroenteritis and environmental contamination in Tunisia.

Pursuing our research on AiV epidemiology, in this paper,

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we report the first seroepidemiological survey of antibodies to AiV in the Tunisian population. One thousand randomly chosen sera from Tunisian individuals were analyzed by an enzyme-linked immunosorbent assay (ELISA) for immunoglobulin G (IgG) specific to AiV. We performed a statistical analysis of IgG antibody levels according to age, and we also looked for AiV-specific immunoglobulin M (IgM) antibodies. We combined the serologic results with clinical and virological data in order to better understand the epidemiology and the role of AiV as a pathogenic agent implicated in gastroenteritis.

MATERIALS AND METHODS

Serum samples. A total of 1,000 sera were randomly collected from Tunisian subjects who consulted at the University Hospital of Monastir, Tunisia, between January and February 2007. Serum samples were selected from patients grouped into the following eight 10-year age ranges: 6 months to 10 years ($n = 106$; 10.6%), 11 to 20 years ($n = 156$; 15.6%), 21 to 30 years ($n = 129$; 12.9%), 31 to 40 years ($n = 118$; 11.8%), 41 to 50 years ($n = 115$; 11.5%), 51 to 60 years ($n = 130$; 13%), 61 to 70 years ($n = 129$; 12.9%), and 71 to 89 years ($n = 117$; 11.7%). In our study, the population was homogeneous. After their arrival in the laboratory, the sera were immediately stored at −20°C.

Cell culture and viruses. AiV strain A846/88 was kindly provided by T. Yamashita. Vero cells were grown in minimal essential medium (MEM) (Gibco) supplemented with 10% fetal calf serum (Eurobio), 1% Eagle's nonessential amino acids (100×; Eurobio), 1% glutamine (200 mM; Sigma), and 1% antibiotics (penicillin, 5,000 UI/ml; streptomycin, 5,000 μg/ml).

As previously described (5), AiV was grown on Vero cells (at 37°C and 5% CO_2). Viral antigen was prepared by clarification of cell lysates (2,465 × *g*; 20 min; 4°C) and then titrated at 10^7 50% tissue culture infective doses ($TCID_{50}$)/ml and stored at −80°C.

Serological study. The detection of AiV-specific antibodies was performed by ELISA. Mock-infected and infected (Aichi) Vero cells were prepared identically, and each sample was also tested on Aichi and mock antigens. A 96-well microtiter plate (Maxisorp, catalog no. 469949; Nunc) was coated with antigen diluted at 1/20 in phosphate-buffered saline (PBS) (pH 7.4) for 1 h at 37°C (100 μl in each well). Then, the wells were blocked with 150 μl of 3% dry skim milk in PBS (Instant Skimmed Milk powder; catalog no. 2920990) at 37°C for 30 min. The supernatants (blocking reagents) were rejected, and 1/100 dilutions of serum samples were distributed into wells and incubated for 30 min at 37°C. The plate was washed 5 times with PBS-Tween 20 (0.1%). Then, 100 μl of horseradish peroxidase (HRP)-conjugated mouse anti-human IgG (catalog no. 9040-05; Southern Biotech) was dispensed into the wells (1/20,000 in PBS), and the plate was incubated at 37°C for 30 min. This was followed by 5 rinses. Enzymatic activity was revealed by the TMB peroxidase substrate (3,3′,5,5′-tetramethylbenzidine [KPL; Eurobio]). The reaction was blocked after 10 min by 50 μl of stop solution (1 N H_2SO_4; Bio-Rad), and the optical absorbance was read at 450 nm with 620 nm as a reference.

In order to align the optical densities (ODs) of samples, we added, in each run, the same negative (human serum with an absorbance value below that of PBS) and positive (a human serum sample with a high antibody titer) calibrators to mock and Aichi plates. We calculated the mean absorbance given on Aichi plates by the positive calibrator for each run. We then determined, for each run, the deviation of its calibrator to this mean in order to align the absorbance values of each serum in each run. The cutoff was determined on the basis of backgrounds obtained on the mock plate. Briefly, the OD was redressed as described above by means of a negative calibrator, and the distribution of the corrected ODs was analyzed, showing that 95% of the samples were below an OD of 0.125. Thus, we considered positive a sample with an absorbance value greater than 0.125 on viral antigen. Signals obtained by this ELISA have been previously shown to be well correlated with those given by a neutralization test (5).

Serological detection of a recent episode of AiV infection. The diagnosis of recent AiV infection was based on the detection of specific IgM antibodies. In this work, 72 ELISA-positive sera from children aged 6 months to 10 years ($n = 106$) were randomly selected and assayed for IgM antibodies specific for AiV by a direct immunofluorescence assay (IFA) on infected Vero cells. Briefly, serum (1/50 in PBS) was added to slides supporting Aichi-infected Vero cells and incubated for 30 min at 37°C. After 4 rinses with PBS, a fluorescein-conjugated anti-human IgM (Bio-Rad) was added and incubated for 30 min at 37°C. The slides were then rinsed as described above and observed with a microscope under UV light. Positive sera showed a typical picture of infected cells containing cytoplasmic granular inclusions.

In order to avoid false-positive results, each positive serum was assessed with the Fidis Rheuma-RF kit (Biomedical Diagnostics, Marne la Vallee, France), which detects rheumatoid factors (RF) (anti-IgG IgM). This assay was performed according to the manufacturer's instructions.

Stool samples and clinical data. A prospective study was previously conducted in Tunisia from January 2003 to April 2007 on stool samples collected from 788 children (413 males and 375 females) under 12 years of age who suffered from acute gastroenteritis. Four hundred eight samples were collected from children within 48 h following their hospitalization for acute gastroenteritis in Monastir University Hospital (inpatients), and 380 samples were collected from children presenting in the dispensaries for gastrointestinal symptoms (outpatients). The samples were screened for routine bacterial agents and parasites and then stored at −20°C for further analyses. Cases were identified by reviewing hospital admission logs for demographic characteristics of the patients (name, age, sex, etc.) and symptoms. Clinical data involving such disease manifestations as fever, vomiting, abdominal pain, or bloody diarrhea were collected for all patients. Severity criteria, such as the duration of the diarrhea, number of stools or bouts of vomiting, range of body temperature, degree of dehydration, capillary refill time (CRT), and presence of skin blotches, were determined for all hospitalized children as previously documented (11).

Statistical analyses. Statistical analyses were performed with STATA software (version 8). Seroprevalence levels were compared by means of 95% confidence intervals established using the binomial exact method. Distribution of ELISA-positive IgG levels (anti-AiV IgG antibodies) for each age group were analyzed by the Kruskall-Wallis test and nonparametric trend test. *P* values of ≤0.05 were considered significant.

RESULTS

Seroprevalence of AiV antibodies. The seroprevalence of specific antibodies to AiV was determined in a total of 1,000 human serum samples randomly collected from Monastir, Tunisia, using an indirect enzyme-linked immunosorbent assay. Our survey showed a high seroprevalence of AiV in the Tunisian population aged from 6 months to 89 years. Among the 1,000 analyzed sera, 917 (92%) were positive, containing IgG antibodies to AiV. Based on the ELISA results, the seroprevalence was calculated for each age group (Fig. 1): the prevalence of antibodies to AiV increased with age, rising from 68.8% (6 months to 10 years) to about 100% (71 to 89 years).The seroprevalence of AiV antibodies progressed statistically with age; in the 21- to 30-year age group, the seroprevalence of AiV antibodies was significantly different ($P < 0.05$.) from those of the preceding age groups (6 months to 10 years and 11 to 20 years). The seroprevalence showed no significant variations from 31- to 40-year-old to 89-year-old patients ($P < 0.05$).

Age distribution of AiV-specific IgG antibody levels. We studied the distribution of IgG ELISA-positive levels among age groups in the Tunisian population (Fig. 2). The statistical analyses of our data showed that the distribution of signal intensities of AiV-specific IgG antibodies among age groups was not homogeneous. Logistic regression analysis was used to compare the 917 OD values by using as an age group reference 24 babies <1 year of age. Our data show that IgG antibody levels increased with age: in young children (1 to 10 years old) and in the 11- to 20-year-old group, IgG levels were low, and no significant difference was observed between these age groups and our reference group ($P = 0.221$ and $P = 0.794$, respectively), whereas, starting from the 21- to 30-year age group, the difference was statistically significant (Kruskall-Wallis test; $P = 0.01$) compared to the reference group. We also found that the rate of significance tended to increase

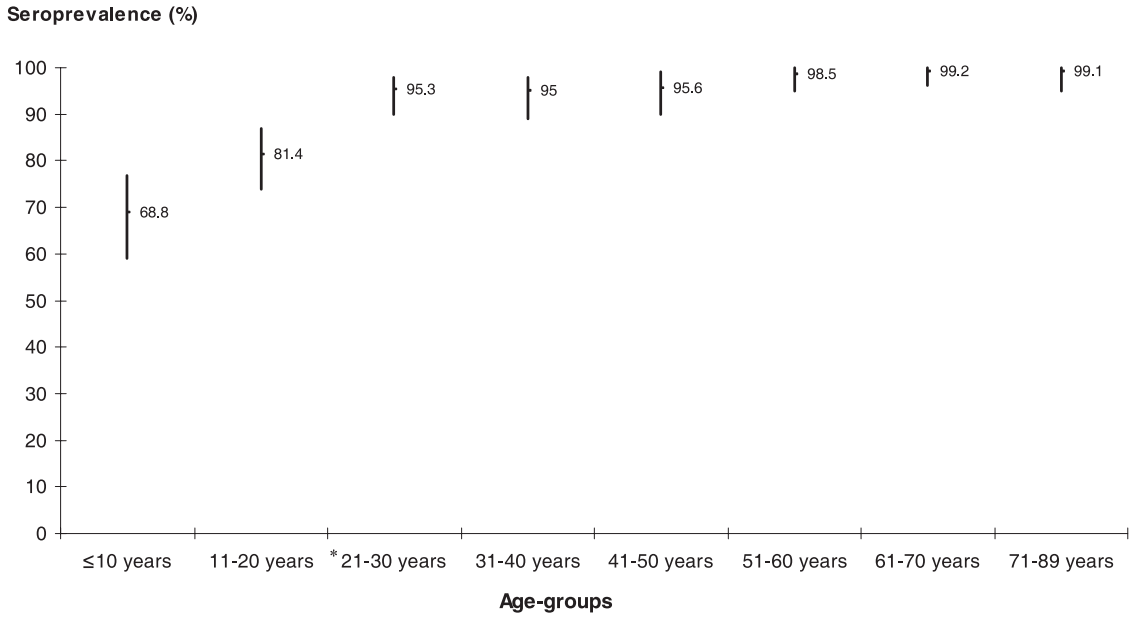

FIG. 1. Age distribution of AiV antibody seroprevalences in a panel of 1,000 randomly selected sera from a Tunisian patient population (the bars represent 95% confidence intervals). *, starting with this age group, the difference from preceding age groups was statistically significant ($P < 0.05$).

according to the age (e.g., $P = 0.002$ for 41 to 50 years and $P = 0.001$ for 51 to 60 years).

Detection of specific IgM antibodies to AiV. In this work, among 72 ELISA-positive sera (IgG antibodies to AiV strains) randomly selected from children aged 6 months to 10 years ($n = 106$), 5 sera were positive for IgM antibodies by IFA (5 children 6 months and 3, 5, 6, and 10 years old). These 5 sera were tested with the Fidis Rheuma-RF kit, and all of them showed negative results (RF < 25 IU/ml).

Virological and clinical data for AiV in Tunisia. Our serological data were combined with those of a clinical survey previously documented in Tunisia between January 2003 and April 2007 involving children suffering from gastroenteritis symptoms (12). In this clinical study, stool samples were all negative for bacterial pathogens and parasites (11). Our previous data showed that 32 samples were positive for AiV. Among monoinfections by Aichi virus ($n = 25$), 18 were detected in stool samples from children suffering from severe diarrhea requiring hospitalization (inpatients) (Table 1). We observed that the ages of children monoinfected by an AiV strain were between 3 months and 9 years.

Among these 18 AiV monoinfection cases, 9 children had

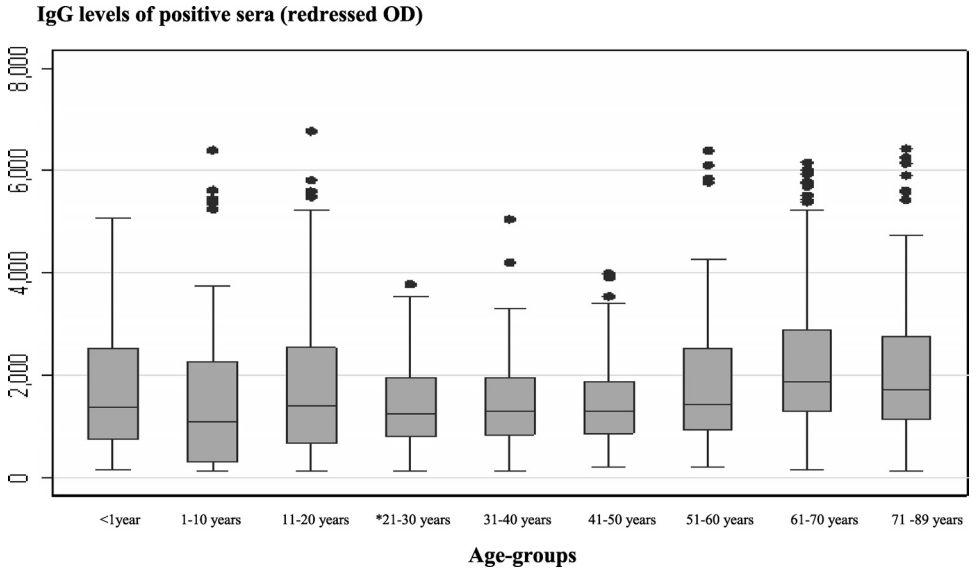

FIG. 2. Serum reactivity against AiV. Shown is a box plot for each age group detailing the distribution if IgG levels as measured by ELISA. *, starting with this age group, the difference from the reference age group was statistically significant (Kruskal-Wallis test; $P < 0.01$).

TABLE 1. Epidemiological and clinical data for children suffering from gastroenteritis symptoms and monoinfected by AiV

Patient no.	Collection date (day/mo/yr)	Age (mo)	Sex[a]	Diarrhea duration (no. of days)	Stool (no./day)	Vomiting (no./day)	Fever[b] (°C)	Abdominal pain	Anemea	Dehydration (degree)[c]	CRT (s)	Skin blotches	Status[d]
333	14/02/2003	10	M	7	5	3	38.5	0	0	1	<3	0	1
4549	21/10/2003	15	M	5	4	0	36	0	0	0	<3	0	1
26512	22/11/2003	29	F	4	5	4	39.7	0	0	0	<3	0	1
27467	05/12/2003	3	M	6	4	1	36.8	0	0	2	<3	0	1
27581	08/12/2003	17	F	2	2	3	37.2	1	0	0	<3	0	1
1633	24/01/2005	9	F	2	3	3	37.4	0	0	0	<3	0	1
2403	31/01/2005	13	M	6	4	3	40	0	0	3	4	1	1
7599	02/04/2005	8.5	M	8	3	4	37.6	0	0	2	<3	0	1
8407	12/04/2005	11	M	6	5	3	36.8	0	0	1	<3	0	1
24459	15/10/2005	4	F	4	6	0	37.5	0	0	2	3	0	1
24953	21/10/2005	11	F	6	7	3	37.5	0	1	2	<3	0	1
25172	24/10/2005	24	M	2	3	3	37	0	0	1	<3	0	1
26035	05/11/2005	108	F	8	9	4	39.2	0	0	0	<3	0	1
27253	18/11/2005	4	M	6	10	3	37	0	0	2	<3	0	1
28669	06/12/2005	7	M	9	5	3	38.5	0	0	2	<3	0	1
30004	20/12/2005	13	M	4	8	5	37.4	0	0	1	<3	0	1
9406	19/04/2006	16	F	9	4	4	37	0	1	0	<3	0	1
20358	13/08/2006	6	F	14	5	4	39	0	0	2	>5	1	Death

[a] M, male; F, female.
[b] Fever was defined as a temperature of ≥37.5°C.
[c] 0, absence of signs of dehydration (normal); 1, mild dehydration (<5%); 2, moderate dehydration (5 to 10%); 3, severe dehydration (>10%).
[d] Status on leaving the hospital. 1, good health (good rehydration and absence of diarrhea and/or vomiting).

fever ($T \geq 37.5$°C), 4 of whom (between 6 months and 9 years old) had fever of ≥39°C; 15 children had CRTs of <3 s, and 3 had CRTs of ≥3 s (3, 4, and >5 s). Of the 18 hospitalized pediatric patients, 17 essentially recovered (good rehydration and absence of diarrhea and/or vomiting). The other was a 6-month-old girl who died during hospitalization. She presented several criteria of severity: diarrhea lasting for 14 days with a frequency of 5 stools/24 h, vomiting (4 episodes/24 h), and fever (39°C), and she suffered from dehydration (degree 2; 5 to 10%). Her CRT was >5 s, and skin blotches were observed. At her admission on 13 August 2006, this child suffered from gastroenteritis symptoms, and after 3 days of hospitalization, she died on 15 August 2006. Our clinical data also showed that among these 18 children monoinfected by the AiV strain, two children with gastroenteritis symptoms had anemia.

It is noteworthy that in the only patient hospitalized for acute gastroenteritis who presented a mixed infection with AiV, the diarrheic stools were also contaminated by rotavirus. This 28-month-old child died during his hospitalization in the pediatric service. At the time of admission, he suffered from diarrhea and vomiting; the diarrhea lasted 5 days, with 4 stools per day and 5 vomiting episodes per day.

DISCUSSION

To our knowledge, this is the first study on the African continent, and especially in Tunisia, to analyze the seroprevalence of Aichi virus. Our results show a high prevalence of antibodies against AiV in the Tunisian population (92%), higher than observed in previous studies conducted in France (77%), Germany (76%), and Japan (55%) (5, 7, 16). Of the 1,000 individuals in our study, 83 (8.3%) remained nonreactive for IgG antibodies, suggesting that at the time of testing, they had had no contact with AiV.

The distribution of seroprevalence according to age groups was not homogeneous. The seroprevalence was age dependent, increasing with age from 68.8% (6 months to 10 years) to about 100% (71 to 89 years). This progression has been observed in countries in Europe and in Japan (5, 7, 16; J. Buesa, personal communication).

Our data are the first to show such a high seroprevalence (about 69%) in young children (<10 years old). Indeed, previous reports showed a high seroprevalence in adolescents and young adults but limited seroprevalence in younger children (7% in Japan and 25% in France), showing that in these countries, AiV seems to primarily infect older persons. Thus, gastroenteritis due to AiV was suggested to mainly affect persons aged 15 to 34 years (5, 16). For babies, maternal antibodies could create a bias in seroprevalence studies (5, 7). However, in our work, we measured the seroprevalence in the first age group by removing children less than 1 year old ($n = 24$), and we did not find any significant difference between these two sets. This suggests that positive children have been infected by AiV and produce specific antibodies in their sera.

The differences in patterns of IgG antibody distribution in these countries could indicate variation in AiV epidemiology.

In Tunisia, a seroprevalence study showed no more progress in the antibody rate in adults and no significant variation from 21- to 30-year-old to 89-year-old patients (with a plateau at around 95%). These data suggest that seroconversions due to first infection by AiV occurred in childhood or adolescence, and in any case, before the age of 30 years. This seroprevalence profile partly corresponds to that observed in several countries: seroconversions occur before the age of 40 in France (84% of positive sera at 30 years of age) (5), as in Japan (83.3% of positive cases at 35 years of age) (16); in Germany, primoinfection occurs before the age of 20 (86% of positive sera at 15 years of age); in Spain, 60% of the population is infected

before 20 years of age and 93% before 40 years of age (J. Buesa, personal communication). The high level of seroprevalence in adults shown in several reports suggests widespread exposure of populations to AiV (5, 7, 16).

In this work, analysis of positive IgG antibody levels by age showed that the signal intensity of IgG antibodies in positive sera (n = 917) was correlated with age. Indeed, the IgG levels of specific anti-AiV antibodies increased gradually and statistically significantly with age; the youngest patients had lower IgG antibody levels, and starting with the 21- to 30-year age group, the difference was statistically significant (Kruskall-Wallis test; $P = 0.01$) compared to patients <1 year of age. These results show that sera were more reactive in persons older than 30 years, which indicates an anamnestic humoral response due to reinfections, even asymptomatic ones.

Among 72 sera tested for IgM antibodies, 5 were positive, and all of them showed negative results in a rheumatoid factor test (anti-IgG IgM), confirming that these IgMs were specific aginst AiV. As IgM antibodies are the serological markers of recent primary infection, their presence in sera indicates the possibility of a recent infection for the 5 corresponding children in the survey, even though such antibodies might be present in the serum up to about 18 months after the first infection (2, 3).

It is interesting to find sera positive for specific AiV IgM antibodies. Indeed the detection of these IgMs specific for AiV has been very rare, as T. Yamashita documented some AiV infections without any detection of anti-AiV IgM during nine outbreaks of gastroenteritis (16). Also, the French study reported no IgM among positive IgG sera even at 1/10 dilution (5).

In our previous clinical and virological study (12), AiV genomic detection was higher than usually documented: it was found in 32 (4.1% of diarrheic stools) cases, with a high proportion of monoinfection (25; 78.1% of AiV infections) and a high frequency of hospitalizations among the monoinfections (18 versus 7 outpatients; $P = 0.04$). The 18 children hospitalized presented with gastroenteritis symptoms, especially diarrhea, fever, and vomiting. Severity criteria, such as dehydration, CRT, the presence of skin blotches, and even death, were observed in these children monoinfected by AiV. These data support the role of AiV in pediatric gastroenteritis in Tunisia as a real pathogenic agent virulent enough to require hospitalization. These previous results are concordant with those from our serological analysis showing a high prevalence of IgG anti-AiV antibodies in children ≤10 years old (68.8%), confirming frequent contact with AiV infection in this age group.

Although AiV pathogenicity was considered doubtful for a long time, and AiV was evaluated as a virus that was not dangerous, causing asymptomatic infections or symptoms not severe enough to require medical attention (13, 18, 19), our data prove that the virus is implicated in severe diarrhea causing hospitalization of children. Thus, the profile of AiV infection seems to be different in Tunisia, and serological and clinical presentation could reinforce ideas concerning the pathogenicity of this emerging virus. Similarly, a recent study conducted in Hungary showed that AiV was detected in a child with clinical symptoms of diarrhea, fever, and purulent conjunctivitis and respiratory symptoms (10).

This work is the first to establish a correlation between the high seroprevalence and the high frequency of AiV isolation and shows that both clinical and virological features of AiV differ between Tunisian and Japanese or European populations, with all other studies showing discrepancies between the relatively high seroprevalence (5, 7, 16; J. Buesa, personal communication) and the low AiV detection rate in sporadic cases and outbreaks of acute gastroenteritis (1, 7, 8, 10, 13, 17).

Moreover, for Tunisian children monoinfected by Aichi virus (12), there was no history of any travel abroad or eating of seafood before the onset of their illness. In addition, in Tunisia, seafood is not commonly consumed but is generally intended for export. Thus, oysters and shellfish can be excluded as principal sources of general AiV exposure, as previously reported (15). These data suggest that AiV can be transmitted in other ways. We think that, like other enteric viruses, AiV is probably transmitted through the fecal-oral route, directly by person-to-person contact or indirectly via fomites or contaminated food or water, especially since AiV strains were detected at high frequency in raw and treated sewage in Tunisia (our unpublished data).

In conclusion, according to our previous results and this serological study, we can consider AiV as an emerging pathogen, very prevalent and requiring diagnosis and study. AiV may be an agent of considerable importance, not only in Asian and European countries, but also in Africa, and further studies are needed to assess the pathogenesis, immunology, and etiological role of AiV in human infection. Our work extends the findings on AiV seroprevalence rates in European and Asian countries to the African continent, in Tunisia. The overall results are similar, confirming infection patterns and adding to our knowledge about the epidemiology of an emerging and understudied virus.

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We declare that we have no financial conflict of interest related to the manuscript and with regard to carrying out the study.

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